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- (71) Applicant (for all designated States except US): CAR-BOZYME OY [FI/FI]; Vellamonkatu 29 D 41, FIN-00550 Helsinki (FI).
- (72) Inventors; and
- (75) Inventors/Applicants (for US only): FENEL, Fred [FI/FI]; Vellamonkatu 29 D 41, FIN-00550 Helsinki (FI). TURUNEN, Ossi [FI/FI]; Männiköntie 23 B 7, FIN-03400 Vihti (FI). LEISOLA, Matti [FI/FI]; Retkikuja 2, FIN-02300 Espoo (FI).
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(54) Title: METHOD TO IMPROVE THE STABILITY AND BROADEN THE PH RANGE OF FAMILY G/11 XYLANASES

(57) Abstract: The present invention relates to protein engineering, and concerns especially family G/11 xylanases, and genes encoding said enzymes. In specific, the invention concerns *Trichoderma reesei* XYNII gene, which codes for endo-1,4- $\beta$ -xylanase (EC 3.2.1.8). The invention describes how site-directed mutagenesis can be used to improve the properties of an enzyme to match the industrial conditions where it is used. Protein engineering can be used to improve thermoactivity and thermostability of xylanases, as well as to broaden their pH range.

## Method to improve the stability and broaden the pH range of family G/11 xylanases

### Field of the invention

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This invention relates to protein engineering, and concerns especially family G/11 xylanases, and genes encoding said enzymes. In specific, the invention concerns *Trichoderma reesei* XYNII gene, which codes for endo-1,4- $\beta$ -xylanase (EC 3.2.1.8). The invention describes how site-directed mutagenesis can be used to improve the properties of an enzyme to match the industrial conditions where it is used. Protein engineering can be used to improve thermoactivity and thermostability of xylanases, as well as to broaden their pH range.

### Background of the invention

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Xylanases are glycosyl hydrolases which hydrolyse  $\beta$ -1,4-linked xylopyranoside chains. Xylanases have been found in at least a hundred different organisms. Together with other glycosyl hydrolases they form a superfamily which includes more than 40 different enzyme families (Henrissat and Bairoch, 1993). Family 11 (previously G) xylanases are defined by the similarities in their gene sequences, protein structures, and catalytic mechanisms. Common features for the members of this family are high genetic homology, a size of about 20 kDa, and a double displacement catalytic mechanism (Tenkanen *et al.*, 1992; Wakarchuk *et al.*, 1994).

25 The family 11 xylanases mainly consist of  $\beta$ -strands which form two large  $\beta$ -sheets, and of one  $\alpha$ -helix. These form a structure that resembles a partly-closed right hand, wherein the  $\beta$ -sheets are called A- and B-sheet. (Törrönen & Rouvinen, 1997). The family 11 xylanases have special interest in industrial applications, because their structure is stable, and they are not susceptible to protease activity. In addition, xylanases can be produced economically on an industrial scale. *Trichoderma reesei* is known to produce three different xylanases of which xylanases I and II (XynI and XynII) are the best characterized (Tenkanen *et al.*, 1992). XynI has a size of 19 kDa, and compared to XynII it has low isoelectric point and pH optimum (pI 5.5, pH 3-4). XynII has a size of 20 kDa and it has a pI of 9.0 and a pH optimum of 5.0-5.5 (Törrönen and Rouvinen, 1995).

35

The most important industrial applications of xylanases are pulp bleaching, modification of textile fibres, and biomass modification to improve its digestion in animal feeding (Prade, 1996). A common nominator in all these applications is the extreme conditions

which face the enzyme. High temperatures, and pH which substantially differs from the optimal pH of many xylanases decrease the effective utility of the presently available xylanases in industrial applications.

5 In feed applications the enzyme faces high temperature conditions for a short time (e.g. 2 - 5 min at 90 °C) during feed preparation. However, the actual catalytic activity of the enzyme is needed at lower temperatures (e.g. ~37 °C). Consequently, the enzyme should not be inactivated irreversibly at high temperatures, while it has to be active at relatively low temperatures.

10

In pulp bleaching the material coming from alkaline wash has a high temperature (> 80 °C) and pH (> 10). None of the commercially available xylanases survives these conditions. The pulp must be cooled and the alkaline pH neutralized in order to treat the pulp with presently available xylanases. This means increased costs. Protein engineering  
15 has been used – sometimes successively – to stabilise xylanases to resist the denaturing effect of the high temperature and pH.

Several thermostable, alkaliphilic and acidophilic xylanases have been found and cloned from thermophilic organisms (Bodie *et al.*, 1995; Fukunaga *et al.*, 1998). However,  
20 production of economical quantities of these enzymes has in most cases proved to be difficult. Therefore the *T. reesei* xylanase II, which is not as such thermostable, is in industrial use because it can be produced at low cost in large quantities. As an alternative for isolating new xylanases, or developing production processes, one can envisage engineering the presently used xylanases to be more stable in extreme conditions.

25

The stability of *Bacillus circulans* xylanase has been improved by disulfide bridges, by binding the N-terminus of the protein to the C-terminus and the N-terminal part of the  $\alpha$ -helix to the neighbouring  $\beta$ -strand (Wakarchuk *et al.*, 1994). Also Campbell *et al.* (1995) have modified *Bacillus circulans* xylanase by inter- and intramolecular disulfide bonds in  
30 order to increase thermostability. On the other hand, the stability of *T. reesei* xylanase II has been improved by changing the N-terminal region to a respective part of a thermophilic xylanase (Sung *et al.*, 1998). In addition to the improved thermostability, the activity range of the enzyme was broadened in alkaline pH. Single point mutations have also been used to increase the stability of *Bacillus pumilus* xylanase (Arase *et al.*,  
35 1993). The influence of mutagenesis on stability has been studied on many other enzymes. By comparing the structures of thermophilic and mesophilic enzymes plenty of information has been obtained (Vogt *et al.*, 1997). Structural information of thermophilic

xylanases has also given information about factors influencing the thermostability of xylanases (Gruber *et al.*, 1998; Harris *et al.*, 1997).

### Summary of the invention

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The present invention relates to xylanases which belong to the family 11 (previously G) glycosyl hydrolases. The invention provides xylanases modified so as to change their thermostability, thermoactivity, and/or broaden their pH range.

10 Various modifications in the *Trichoderma reesei* xylanase structure, either alone or in combinations, result in the changes described in this invention:

(1) the stability of the enzyme is increased by binding of the N-terminal region by disulfide bridges (for example, the bridges formed by the mutation pairs T2C and T28C; P5C and N19C; T7C and S16C; N10C and N29C) to the body of the protein;

15 (2) the C-terminus is stabilised by extension with additional aspartic acid (-191D) which forms a salt bridge with arginine 58 (lysine 58 in the wild-type enzyme has been changed to arginine (K58R));

(3) the stability of the enzyme is increased by binding the  $\alpha$ -helix by a disulfide bridge to the body of the enzyme (e.g. L105C and Q162C);

20 (4) point mutations have been made at different positions to improve the stability of xylanase (N11D, T26R, G30H, N67R, N97R, A132R, N157R, A160R, T165N, M169H, S186R).

In specific, the present invention provides a modified *Trichoderma reesei* xylanase in  
25 which the amino acids T2 and T28 have been changed to cysteines, K58 has been changed to arginine, and to the C-terminus of the enzyme an aspartic acid has been added (+191D), thereby forming a disulfide bridge between the amino acids T2C and T28C, and a salt bridge between the amino acids K58R and +191D.

### 30 Brief description of drawings

**Figure 1.** A set of oligonucleotides used in the mutagenesis of xylanase (codon changes underlined). The sequences are also given in the appended Sequence Listing as sequences 1 to 12.

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**Figure 2.** A graph presenting the effect of the mutations T2C, T28C, K58R, and +191D on the thermal optimum of *T. reesei* XynII (WT = wild-type enzyme; Y5 = the mutated *T. reesei* XynII).

**Figure 3.** A graph presenting the effect of the mutations T2C, T28C, K58R, and +191D on the pH-dependent activity of *T. reesei* XynII (WT and Y5 as in Figure 2).

**Figure 4.** A graph presenting the effect of the mutations T2C, T28C, K58R, and +191D on the inactivation of *T. reesei* XynII at different temperatures (WT and Y5 as in Figure 2).

**Figure 5.** A graph presenting the effect of the mutations Q162C and L105C on the inactivation of *T. reesei* XynII at different temperatures (W.t. = wild-type enzyme).

#### Detailed description of the invention

The family G/11 xylanases originating from bacteria, yeast and fungi have common molecular structure. Examples of such xylanases are:

- Aspergillus niger* XynA
- Aspergillus kawachii* XynC
- Aspergillus tubigensis* XynA
- Bacillus circulans* XynA
- Bacillus pumilus* XynA
- Bacillus subtilis* XynA
- Neocallimastix patriciarum* XynA
- Streptomyces lividans* XynB
- Streptomyces lividans* XynC
- Streptomyces thermoviolaceus* XynII
- Thermomonospora fusca* XynA
- Trichoderma harzianum* Xyn
- Trichoderma reesei* XynI, *Trichoderma reesei* XynII
- Trichoderma viride* Xyn

The invention deals with xylanases of the family G/11 with the following common features:

- (i) Enzymes in which the N-terminal sequence is a part of the double-layered  $\beta$ -sheet (in the family 11 xylanases the A- and the B-sheet, (Gruber, *et al.*, 1998)) and in which the first  $\beta$ -strand (in *T. reesei* XynII the amino acids 5-10) or the N-terminal end can be

bound by disulfide bridges either to the adjacent  $\beta$ -strands (in *T. reesei* XynII the amino acids 13-19) or to other neighbouring regions.

(ii). Enzymes in which the C-terminal peptide chain forms a  $\beta$ -strand (in *T. reesei* XynII amino acids 183-190), which is a part of a larger  $\beta$ -sheet and in which the C-terminal region can be bound by disulfide bridges to the adjacent  $\beta$ -strands or by salt bridges to the body of the enzyme.

(iii). Enzymes which have an  $\alpha$ -helix on the other side of the enzyme structure with regard to the catalytic canyon, and wherein said  $\alpha$ -helix or the neighbouring regions can be bound more tightly by a disulfide bridge to the body of the protein.

The *T. reesei* xylanase II has the above mentioned properties and in said enzyme thermostability, pH-stability and thermoactivity can be modified based on these properties. The following changes have been made to the xylanase gene (*XYNII*) of *T. reesei*:

1. By site-directed mutagenesis disulfide bridges are formed in the N-terminal region:

\* Threonines 2 and 28 are changed to cysteines resulting in a disulfide bridge being formed between them (T2C and T28C).

\* Proline 5 and asparagine 19 are changed to cysteines resulting in a disulfide bridge being formed between them (P5C and N19C).

\* Threonine 7 and serine 16 are changed to cysteines resulting in a disulfide bridge being formed between them (T7C and S16C).

\* Asparagine 10 and asparagine 29 are changed to cysteines resulting in a disulfide bridge being formed between them (N10C and N29C).

2. By site-directed mutagenesis, the C-terminus is bound more tightly to the body of the enzyme by adding as a recombinant change one amino acid (e.g. aspartic acid or glutamic acid) to the C-terminus of the xylanase, which then forms a salt bridge from the C-terminus to the body of the enzyme. If appropriate, a suitable amino acid replacement can be made in the body of the protein, so as to enable the formation of a salt bridge.

\* An aspartic acid (+191D) is added to the C-terminal serine (S190). This results in a salt bridge with arginine at position 58, where wild-type lysine has been replaced by arginine (K58R).

3. By site-directed mutagenesis at least one disulfide bridge is formed to stabilise the enzyme in the C-terminal part via the  $\alpha$ -helix or a region near the  $\alpha$ -helix.

5 \* Leucine 105 and glutamine 162 are changed to cysteines resulting in disulfide bridge between them (L105C and Q162C).

4. By site-directed mutagenesis point mutations are made to increase the stability of *T. reesei* xylanase II: N11D, T26R, G30H, N67R, N97R, A132R, N157R, A160R, T165N, M169H, S186R.

10

### Methods of the invention

Production of mutated and recombinant *XYNII* genes were carried out by the following general procedures:

15

#### 1. Expression vector and production of the enzyme

*T. reesei* xylanase II was produced in *E. coli* strains XL1-Blue or Rv308 using the vector pKKtac (VTT, Espoo, Finland) or the vector pALK143 (ROAL, Rajamäki, Finland). *T. reesei* *XYNII* gene was directly cloned by PCR from the cDNA of *T. reesei* to the vector pKKtac (induction of expression by IPTG). Alternatively, the plasmid pALK143 was used which contains *T. reesei* *XYNII* gene. Both of the vectors secrete the xylanase into the medium; the vector pKKtac by pectate lyase (pelB) signal sequence and the vector pALK143 by amylase signal sequence.

#### 25 2. Site-directed mutagenesis and production of recombinant *XYNII* gene

The production of mutated *T. reesei* *XYNII* gene used in the Examples of this application, was effected as follows: Mutations were produced by polymerase chain reaction (PCR) using oligonucleotide primers which contained the sequences for the changed codons. Examples of the used oligonucleotides are given in Figure 1, as well as in the appended Sequence Listing as sequences 1 to 12. PCR using the primers (containing the desired mutation) was carried out by Quick Change method (Stratagene, Westburg, Leusden, The Netherlands) and by generally known methods. PfuTurbo was used as DNA polymerase (Stratagene, La Jolla, Ca, USA). The cloned *E. coli* strains were cultivated on plates containing xylan (birchwood xylan: Sigma, Steinheim, Germany) coupled to Rhemazol Brilliant Blue. The xylanase activity could be seen as halos around the colonies (Biely *et al.*, 1985).

30

### 3. Determination of the activity of xylanases

The xylanase activity of enzyme samples was determined by measuring the amount of reducing sugars released from the hydrolysed xylan fibre. The reducing sugars were measured by DNS-method in 50 mM citrate-phosphate buffer (Bailey *et al.*, 1992).

5 Standard activity determination was carried out at pH 5 and 50 °C.

### 4. Determination of the stability of the enzymes

The stability of the xylanases was tested by measuring the half-life of the modified enzymes at different temperatures. The enzyme was incubated for varying times at 55 or  
10 65 °C and the residual activity was measured as described above. The stability at high temperatures was also measured by incubating the enzymes for 10 min at varying temperatures and subsequently measuring the residual activity by DNS-method. The pH-dependent xylanase activity was measured by determining the enzyme activity in varying pH-values. The temperature optimum of the enzyme was determined by measuring the  
15 activity at varying temperatures (10 min, pH 5). The properties of the mutated enzymes were compared to the wild-type *T. reesei* XynII enzyme.

### Examples of mutations

#### 20 Example 1.

The three-fold mutations (T2C, T28C and K58R) and the recombinant change (+191D) were made in *T. reesei* XynII by using the methods described above. The mutant enzyme was designated as Y5. Said mutant enzyme was expressed in *E. coli*, which was cultivated at +37 °C in shake flasks using Luria Broth as growth medium. After  
25 cultivation the cells were removed by centrifugation and the xylanase secreted into the medium was characterized in varying conditions, as described above. Figure 2 shows the effect of the temperature to the enzyme activity when the mutant Y5 (T2C, T28C, K58R, +191D) and the wild-type (*T. reesei* XynII) enzyme were incubated for 10 min with birchwood xylan in varying temperatures, and the relative amount of the reducing  
30 sugars as released were measured with DNS-method. Said mutations improved the temperature optimum of xylanase by about 15 °C.

#### Example 2.

The three-fold mutant xylanase (T2C, T28C, K58R, +191D) described in Example 1 was  
35 incubated for 10 min in 1% birchwood xylan at 50 °C in citrate-phosphate buffer in varying pH-values. Figure 3 shows the relative amount of reducing sugars as released for the mutant and the wild-type xylanases. The mutations broadened slightly the pH-dependent activity of the enzyme to alkaline direction. The mutant enzyme was more

active than the wild-type enzyme at pH 7 - 8; the activity of mutant enzyme was about 20% higher at pH 8 (50 °C) .

### Example 3.

- 5 The above-mentioned three-fold mutant (T2C, T28C, K58R,+191D) and the wild-type enzyme were incubated for 10 min at varying temperatures. After the incubation the samples were cooled and the residual activity was determined in standard conditions. The wild-type enzyme was completely inactivated already at 55-60 °C. The mutant enzyme retained about 50% of its activity even at 65 °C (Figure 4). Table 1 below  
10 shows the half-lives (T<sub>1/2</sub>) of the mutant (Y5) and the wild-type xylanase at 55 °C and 65 °C.

Table 1.

	pH5	pH8
55 °C		
Y5	stable	stable
Wild-type XynII	~5 min	~2 min
65 °C		
Y5	20 - 25 min	~10 min
Wild-type XynII	40 sec	

15

### Example 4.

- With the above-mentioned methods a disulfide bridge was made (L105C and Q162C) to bind the C-terminus of the  $\alpha$ -helix to the neighbouring  $\beta$ -strand. The enzyme was  
20 produced in *E. coli* and its properties were determined. Figure 5 shows the inactivation of the mutant enzyme at different temperatures compared to the wild-type enzyme. At 55 °C the stability of the mutated enzyme increased about 20-fold, with regard to the wild-type enzyme, whereby the half-life increased from 5 min (the wild-type enzyme) up to about 1,5 hours (the mutated enzyme).

25

## Literature

- Arase, A., Yomo, T., Urabe, I., Hata, Y., Katsube, Y. & Okada, H. (1993). Stabilization of xylanase by random mutagenesis. *FEBS Letters* 316, 123-7.
- 5 Bailey, J. M., Biely, P. & Poutanen, K. (1992). Interlaboratory testing of methods for assay of xylanase activity. *J. Biotech.* 23, 257-270.
- 10 Biely, P., Mislovicova, D. & Toman, R. (1985). Soluble chromogenic substrates for the assay of endo-1,4-beta-xylanases and endo-1,4-beta-glucanases. *Analytical Biochemistry* 144, 142-6.
- Bodie, E., Cuevas, W. A. & Koljonen, M. (1995). In *United States Patent* 5,437,992.
- 15 Campbell, R.L., Rose, D.R., Sung, W.L., Yaguchi, M. & Wakarchuck, W. (1995). In *United States Patent* 5,405,769.
- Fukunaga, N., Iwasaki, Y., Kono, S., Kita, Y. & Izumi, Y. (1998). In *United States Patent* 5,736,384.
- 20 Gruber, K., Klintschar, G., Hayn, M., Schlacher, A., Steiner, W. & Kratky, C. (1998). Thermophilic xylanase from *Thermomyces lanuginosus*: High-resolution X-ray structure and modeling studies. *Biochemistry* 37, 13475-13485.
- 25 Harris, G. W., Pickersgill, R. W., Connerton, I., Debeire, P., Touzel, J. P., Breton, C. & Perez, S. (1997). Structural basis of the properties of an industrially relevant thermophilic xylanase. *Proteins* 29, 77-86.
- 30 Henrissat, B. & Bairoch, A. (1993). New families in the classification of glycosyl hydrolases based on amino acid sequence similarities. *Biochemical Journal* 293, 781-8.
- Prade, R. A. (1996). Xylanases: from biology to biotechnology. *Biotechnology & Genetic Engineering Reviews* 13, 101-31.
- 35 Sung, W. L., Yaguchi, M., Ishikawa, K., Huang, F., Wood, M. & Zahab, D. M. (1998). In *United States Patent* 5,759,840.
- Tenkanen, M., Puls, J. & Poutanen, K. (1992). Two major Xylanases of *Trichoderma reesei*. *Enzyme Microb. Technol.* 14, 566-574.
- 40 Törrönen, A. & Rouvinen, J. (1995). Structural comparison of two major endo-1,4-xylanases from *Trichoderma reesei*. *Biochemistry* 34, 847-56.
- Törrönen, A. & Rouvinen, J. (1997). Structural and functional properties of low molecular weight endo-1,4-beta-xylanases. *Journal of Biotechnology* 57, 137-49.
- 45 Wakarchuk, W. W., Sung, W. L., Campbell, R. L., Cunningham, A., Watson, D. C. & Yaguchi, M. (1994). Thermostabilization of the *Bacillus circulans* xylanase by the introduction of disulfide bonds. *Protein Engineering* 7, 1379-86.

Vogt, G., Woell, S. & Argos, P. (1997). Protein thermal stability, hydrogen bonds, and ion pairs. *Journal of Molecular Biology* 269, 631-43.

## Claims

1. A modified xylanase enzyme of family G/11, having increased thermostability or pH-stability with respect to the corresponding wild-type xylanase, the wild-type enzyme  
5 having been modified by
  - binding the N-terminal region of the enzyme by disulfide bridges to the body of the protein, and/or
  - binding the C-terminal region of the enzyme by salt bridges to the body of the protein,  
or
  - 10 - binding the  $\alpha$ -helix of the enzyme by disulfide bridges to the body of the protein, or
  - making single amino acid mutations in the protein.
2. The modified xylanase according to claim 1, wherein the xylanase is a *Trichoderma reesei* xylanase.  
15
3. The modified xylanase according to claim 2, wherein the xylanase is the *Trichoderma reesei* xylanase II (XynII).
4. The modified xylanase according to any one of the claims 1 to 3, wherein binding the  
20 C-terminal region of the enzyme to the body of the protein has been effected by adding aspartic acid or glutamic acid to the C-terminus so that a salt bridge is formed between said added amino acid and a suitable amino acid in the body of the protein.
5. The modified xylanase according to any one of the claims 1 to 4, wherein binding the  
25 N-terminal region of the enzyme to the body of the protein has been effected by forming a disulfide bridge between the amino acids T2C and T28C of the *Trichoderma reesei* xylanase II (XynII) or between the corresponding amino acids of other xylanases of the family G/11.
6. The modified xylanase according to claim 1, wherein in *T. reesei* xylanase II (XynII)  
30 amino acids T2 and T28 have been changed to cysteines, K58 has been changed to arginine, and to the C-terminus of the enzyme an aspartic acid has been added (+191D), thereby forming a disulfide bridge between the amino acids T2C and T28C and a salt bridge between the amino acids K58R and +191D.  
35
7. The modified xylanase according to any one of the claims 1 to 3, wherein binding the C-terminal region of the enzyme by disulfide bridges to the body of the protein has been effected by binding the C-terminal part of the  $\alpha$ -helix to the adjacent  $\beta$ -strand by a

disulfide bridge between the amino acids L105C and Q162C of *Trichoderma reesei* xylanase II (XynII) or between the corresponding amino acids of other xylanases of the family G/11.

- 5 8. A method for improving the thermostability and/or broadening the pH-range of family G/11 xylanases, comprising carrying out the steps of:
- binding the N-terminal region of the enzyme by disulfide bridges to the body of the protein, and/or
  - binding the C-terminal region of the enzyme by salt bridges to the body of the protein,
- 10 or
- binding the  $\alpha$ -helix of the enzyme by disulfide bridges to the body of the protein, or
  - making single amino acid mutations in the protein.
- 15 9. The method according to claim 8, wherein aspartic acid or glutamic acid is added to the C-terminus so that a salt bridge is formed between said added amino acid and a suitable amino acid in the body of the protein.
- 20 10. The method according to claim 9, wherein in *T. reesei* xylanase (XynII) the additional aspartic acid or glutamic acid forms a salt bridge with the amino acid 58 which has been changed to lysine or arginine.
- 25 11. The method according to claim 8, wherein in *T. reesei* xylanase (XynII) amino acids T2 and T28 are changed to cysteines, K58 is changed to arginine, and to the C-terminus of the enzyme an aspartic acid is added (+191D), thereby forming a disulfide bridge between the amino acids T2C and T28C and a salt bridge between the amino acids K58R and +191D.
- 30 12. The method according to claim 8, wherein single amino acid mutations are made.
13. The method according to claim 12, wherein in *T. reesei* XynII the modification N11D is made.

1/5

T2C	5'-GAGAAGCGCCAGTGCATTTCAGCCCGGC-3'
T28C	5'-GTGACGTACTGCAATGGTCCCGGCGGG-3'
K58R	5'-GGCACCAAGAACAGGGTCATCAACTTCTCGGGC-3'
I91D	5'-TCCATCACCGTCAGCGATTAAAGGGGGCTCTTC-3'
P5C	5'-CCCAGACGATTTCAGTGCGGCACGGGCTACAAC-3'
N19C	5'-CTTCTACTCGTACTGGTGCATGGCCACGGCG-3'
T7C	5'-CGATTCAGCCCGGCTGCGGCTACAACAACGGC-3'
S16C	5'-CAACGGCTACTTCTACTGCTACTGGAACGATGGCC-3'
N10C	5'-CCGGCACGGGCTACTGCAACGGCTACTTCTACTC-3'
N29C	5'-GGCGTGACGTACACCTGCGGTCCCGGCGGGC-3'
L105C	5'-GGCGCCACCAAGTGC GGCGAGGTCACC-3'
Q162C	5'-GCGTGGGCTCAGTGC GGCCGTGACGCTCG-3'

Figure 1

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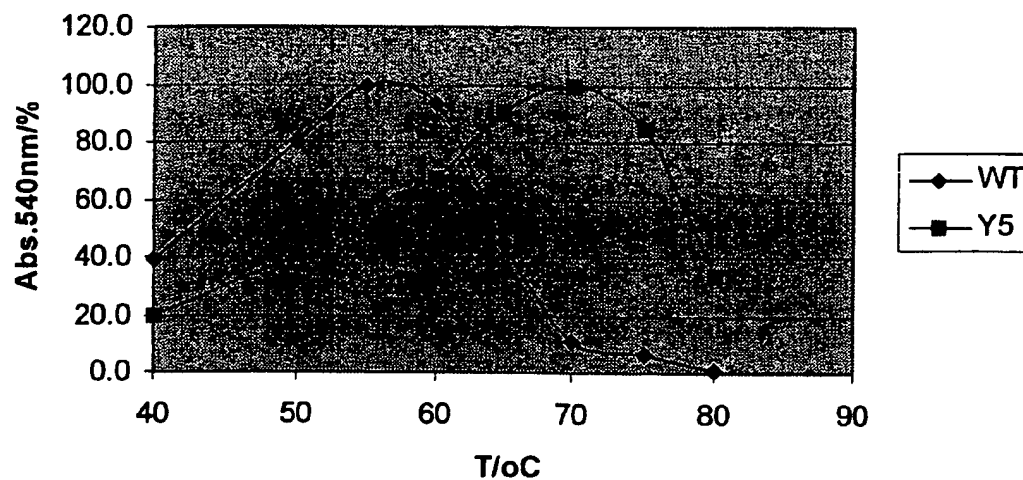
**WT,Y5 enzyme activity**

Figure 2

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## WT,Y5 pH activity

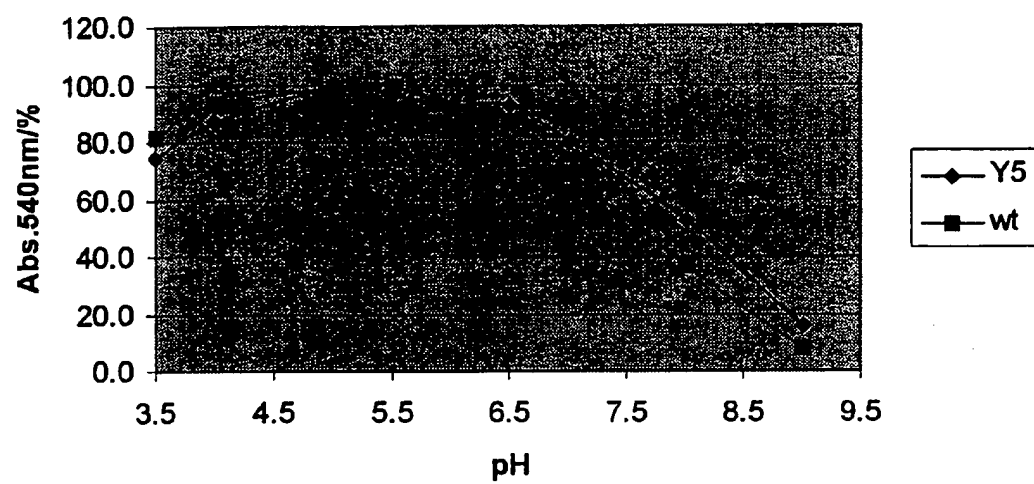


Figure 3

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## WT, Y5 residual activity

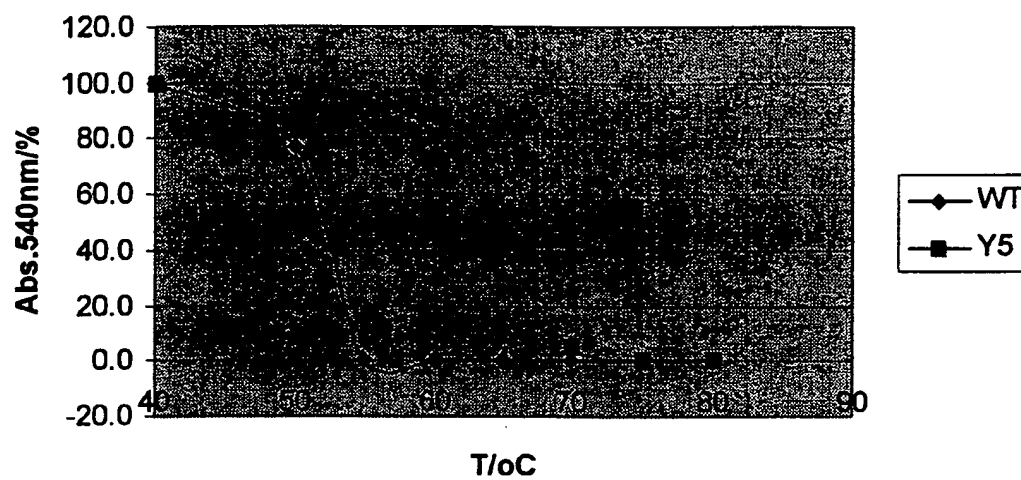


Figure 4

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**WT, disulphide bridge 162-105  
residual activity**

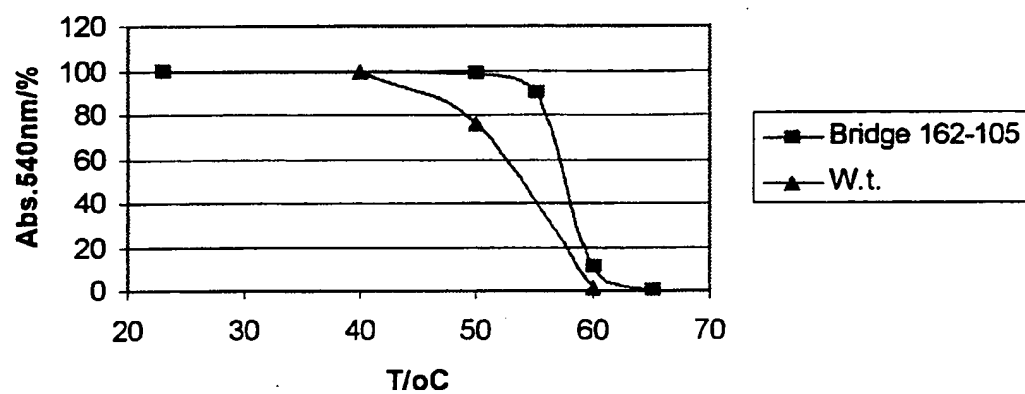


Figure 5

## SEQUENCE LISTING

<110> Carbozyme Oy

<120> Method to improve the stability and broaden the pH range of family G/11 xylanases

<130> 34142

<140>

<141>

<160> 12

<170> PatentIn Ver. 2.1

<210> 1

<211> 27

<212> DNA

<213> Artificial Sequence

<220>

<223> Description of Artificial Sequence: The oligonucleotide used in the mutation T2C

<400> 1

gagaagcgcc agtgcattca gcccggc

27

<210> 2

<211> 27

<212> DNA

<213> Artificial Sequence

<220>

<223> Description of Artificial Sequence: The oligonucleotide used in the mutation T28C

<400> 2

gtgacgtact gcaatgggtcc cggcggg

27

<210> 3

<211> 33

<212> DNA

<213> Artificial Sequence

<220>

<223> Description of Artificial Sequence: The oligonucleotide used in the mutation K58R

<400> 3

ggcaccaaga acagggtcat caacttctcg ggc

33

<210> 4

<211> 33

<212> DNA  
<213> Artificial Sequence

<220>  
<223> Description of Artificial Sequence: The  
oligonucleotide used in the mutation +191D

<400> 4  
tccatcacg tcagcgatta aagggggctc ttc 33

<210> 5  
<211> 32  
<212> DNA  
<213> Artificial Sequence

<220>  
<223> Description of Artificial Sequence: The  
oligonucleotide used in the mutation P5C

<400> 5  
cccagacgat tcagtgcggc acgggctaca ac 32

<210> 6  
<211> 32  
<212> DNA  
<213> Artificial Sequence

<220>  
<223> Description of Artificial Sequence: The  
oligonucleotide used in the mutation N19C

<400> 6  
cttctactcg tactgggtgcg atggccacgg cg 32

<210> 7  
<211> 32  
<212> DNA  
<213> Artificial Sequence

<220>  
<223> Description of Artificial Sequence: The  
oligonucleotide used in the mutation T7C

<400> 7  
cgattcagcc cggctgcggc tacaacaacg gc 32

<210> 8  
<211> 35  
<212> DNA  
<213> Artificial Sequence

<220>  
<223> Description of Artificial Sequence: The

oligonucleotide used in the mutation S16C

<400> 8  
caacggctac ttctactgct actggaacga tggcc 35

<210> 9  
<211> 34  
<212> DNA  
<213> Artificial Sequence

<220>  
<223> Description of Artificial Sequence: The  
oligonucleotide used in the mutation N10C

<400> 9  
cgggcacggg ctactgcaac ggctacttct actc 34

<210> 10  
<211> 31  
<212> DNA  
<213> Artificial Sequence

<220>  
<223> Description of Artificial Sequence: The  
oligonucleotide used in the mutation N29C

<400> 10  
ggcgtgacgt acacctgcgg tcccggcggg c 31

<210> 11  
<211> 27  
<212> DNA  
<213> Artificial Sequence

<220>  
<223> Description of Artificial Sequence: The  
oligonucleotide used in the mutation L105C

<400> 11  
ggcgccacca agtgcggcga ggtcacc 27

<210> 12  
<211> 28  
<212> DNA  
<213> Artificial Sequence

<220>  
<223> Description of Artificial Sequence: The  
oligonucleotide used in the mutation Q162C

<400> 12  
gcgtgggctc agtgcggcct gacgctcg 28

## INTERNATIONAL SEARCH REPORT

International application No.

PCT/FI 00/00877

## A. CLASSIFICATION OF SUBJECT MATTER

IPC7: C12N 9/42

According to International Patent Classification (IPC) or to both national classification and IPC

## B. FIELDS SEARCHED

Minimum documentation searched (classification system followed by classification symbols)

IPC7: C12N

Documentation searched other than minimum documentation to the extent that such documents are included in the fields searched

SE,DK,FI,NO classes as above

Electronic data base consulted during the international search (name of data base and, where practicable, search terms used)

## C. DOCUMENTS CONSIDERED TO BE RELEVANT

Category*	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
X	Protein Engineering, Volume 7, No 11, 1994, Warren W. Wakarchuk et al, "Thermostabilization of the Bacillus circulans xylanase by the introduction of disulfide bonds" page 1379 - page 1386 --	1-12
X	US 5405769 A (ROBERT L. CAMPBELL ET AL), 11 April 1995 (11.04.95) --	1-3,7-8,12
X	US 5866408 A (WING L. SUNG ET AL), 2 February 1999 (02.02.99) --	1-3,8,12-13

☒ Further documents are listed in the continuation of Box C.☒ See patent family annex.

\* Special categories of cited documents:

- "A" document defining the general state of the art which is not considered to be of particular relevance
- "E" earlier application or patent but published on or after the international filing date
- "L" document which may throw doubts on priority claim(s) or which is cited to establish the publication date of another citation or other special reason (as specified)
- "O" document referring to an oral disclosure, use, exhibition or other means
- "P" document published prior to the international filing date but later than the priority date claimed

"T" later document published after the international filing date or priority date and not in conflict with the application but cited to understand the principle or theory underlying the invention

"X" document of particular relevance: the claimed invention cannot be considered novel or cannot be considered to involve an inventive step when the document is taken alone

"Y" document of particular relevance: the claimed invention cannot be considered to involve an inventive step when the document is combined with one or more other such documents, such combination being obvious to a person skilled in the art

"&amp;" document member of the same patent family

Date of the actual completion of the international search

23 January 2001

Date of mailing of the international search report

29-01-2001

Name and mailing address of the ISA/

Swedish Patent Office  
Box 5055, S-102 42 STOCKHOLM  
Facsimile No. +46 8 666 02 86

Authorized officer

Carolina Palmcrantz/EÖ  
Telephone No. +46 8 782 25 00

## INTERNATIONAL SEARCH REPORT

International application No.

PCT/FI 00/00877

C (Continuation). DOCUMENTS CONSIDERED TO BE RELEVANT		
Category*	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
P,X	<p>Protein Science, Volume 9, 2000, Jacques Georis et al, "An additional aromatic interaction improves the thermostability and thermophilicity of a mesophilic family 11 xylanase: Structural basis and molecular study" page 466 - page 475</p> <p>-- -----</p>	1-3,8,12

# INTERNATIONAL SEARCH REPORT

International application No.  
PCT/FI00/00877

## Box I Observations where certain claims were found unsearchable (Continuation of item 1 of first sheet)

This international search report has not been established in respect of certain claims under Article 17(2)(a) for the following reasons:

1. ☐ Claims Nos.:  
because they relate to subject matter not required to be searched by this Authority, namely:
  
2. ☐ Claims Nos.:  
because they relate to parts of the international application that do not comply with the prescribed requirements to such an extent that no meaningful international search can be carried out, specifically:
  
3. ☐ Claims Nos.:  
because they are dependent claims and are not drafted in accordance with the second and third sentences of Rule 6.4(a).

## Box II Observations where unity of invention is lacking (Continuation of item 2 of first sheet)

This International Searching Authority found multiple inventions in this international application, as follows:

**see next sheet**

1. ☐ As all required additional search fees were timely paid by the applicant, this international search report covers all searchable claims.
2. ☒ As all searchable claims could be searched without effort justifying an additional fee, this Authority did not invite payment of any additional fee.
3. ☐ As only some of the required additional search fees were timely paid by the applicant, this international search report covers only those claims for which fees were paid, specifically claims Nos.:
  
4. ☐ No required additional search fees were timely paid by the applicant. Consequently, this international search report is restricted to the invention first mentioned in the claims; it is covered by claims Nos.:

### Remark on Protest

- ☐ The additional search fees were accompanied by the applicant's protest.  
☐ No protest accompanied the payment of additional search fees.

According to PCT Article 34 (3) (a-c) and Rule 13.2, an international application shall relate to one invention only or to a group of inventions linked by one or more of the same or corresponding "special technical features". These special technical features are features, that define a contribution which each of the inventions make over the prior art.

The problem, which the claimed invention aims to solve, is how to increase the thermostability or pH-stability of xylanase enzymes of family G/11. This problem is, according to the application, solved, by modifying the amino acid sequence of the enzyme in different ways, in order to create new interactions such as disulfide or salt bridges. Through for example US patent 5,405,769 it is however known to modify the amino acid sequence of xylanses and thereby creating disulfide bridges in order to increase the thermostability or pH-stability of the enzyme. The application discloses different solutions of the named technical problem. The search has not revealed any document, which can provide a common concept, shared by these solutions, and the application is considered to show a lack of unity. The International Searching Authority has arrived at the following principle of division:

Invention A: Creation of disulfide bridges binding the N-terminal region or the  $\alpha$ -helix to the body of the enzyme. Claims 1-3 (partially), 5-8 (partially) and 11 (partially).

Invention B: Binding the C-terminal region to the body of the enzyme by salt bridges. Claims 1-3 (partially), 4, 6 (partially), 8 (partially), 9-10 and 11 (partially).

Invention C: Making single amino acid substitutions. Claims 1-3 (partially), 8 (partially) and 12-13.

However, the search report has covered all these three inventions.

**INTERNATIONAL SEARCH REPORT**  
Information on patent family members

27/12/00

International application No.  
PCT/FI 00/00877

Patent document cited in search report			Publication date	Patent family member(s)		Publication date
US	5405769	A	11/04/95	WO	9424270 A	27/10/94
<hr/>						
US	5866408	A	02/02/99	CA	2210247 A	09/03/98
				EP	0828002 A	11/03/98
				JP	10179155 A	07/07/98
				NZ	328680 A	27/04/98
				US	5759840 A	02/06/98
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